Structural basis of CB118 metabolism by bacterial cytochrome P450 monooxygenase and its mutants with perfluorocarboxylic acids

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Introduction

Polychlorinated biphenyls (PCBs) classified as persistent organic pollutants show high toxicity, long distance mobility, and persistency in the environment because of their chemical stability and lipophilicity. Despite many countries have banned the use of PCBs since 1970s, the contamination of PCBs is still found throughout the environment of the world. PCBs are accumulated in animals via the food chain, and their toxicity has become social concerns. 2,3',4,4',5-Pentachlorobiphenyl (CB118, Figure 1) is the most abundant PCB congener in the environment including river sediments, algae, plants, and fish.

Mammalian cytochrome P450 (P450 or CYP) monooxygenases are responsible for PCB metabolism in humans [1]. The previous studies on metabolism of CB118 revealed that human CYP2B6 and rat CYP2B1 primarily metabolized to 3-hydroxy (OH)-CB118 [2]. Moreover, rat CYP1A1 primarily metabolized CB118 to 4-OH-2,3,3',4',5-pentachlorobiphenyl (4-OH-CB107). In contrast, P450BM3, isolated from the soil bacterium *Bacillus megaterium*, catalyzes the oxidation of long chain fatty acids, and it shows the highest catalytic activity among P450s [3]. However, there are no reports on PCB metabolism by P450BM3.

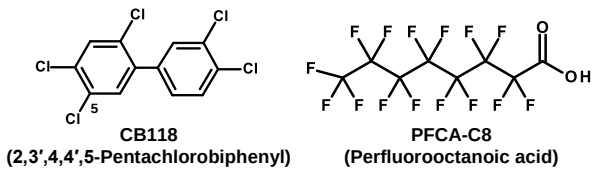

Figure 1 Structures of CB118 and PFCA-C8

In the previous study, a new reaction system to metabolize unnatural substrates by wild-type P450BM3 (WT) with decoy molecules was developed [4]. This system used decoy molecules, perfluorocarboxylic acids (PFCAs, Figure 1), as dummy substrates to trigger the reaction and to anchor the target substrates in the reaction space. It was reported that WT metabolized unnatual substrates such as short alkane and benzene using this reaction system [5].

In this study, we show the metabolism of CB118 by WT and its mutants with assistance of PFCAs as decoy molecules. Furthermore, we try to clarify the difference of metabolic reactions between WT and its mutants by *in silico* study.

Materials and method

CB118 was purchased from AccuStandard (New Haven, CT). CB118 was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 6.12 mM. ^{13}C-labeled hydroxylated (OH)-PCBs used as internal standards and [$^{13}C_{12}$]-2,3′,4′,5-tetrachlorobiphenyl used as a syringe spike were purchased from Wellington Laboratories (Guelph, Canada). MPCB-MXA containing 4′-methoxy (MeO)-2,3,4,5-tetrachlorobiphenyl (4′-MeO-CB61) and 4′-MeO-2,3′,4,5,5′-pentachlorobiphenyl (4′-MeO-CB120) was purchased from Wellington Laboratories. PFCAs containing perfluorohexanoic acid (PFCA-C6), perfluoroheptanoic acids (PFCA-C7), perfluorooctanoic acid (PFOA, PFCA-C8), perfluorononanoic acid (PFCA-C9), perfluorodecanoic acid (PFCA-C10), and

perfluoroundecanoic acid (PFCA-C11) were purchased from Wellington Laboratories.

P450BM3 (*CYP102A1*) *WT* gene was expressed on the pT7Bm3HdZ vector in *Escherichia coli*. A mutation at F87A or A264G was introduced to the *WT* gene by a site directed mutagenesis using primers with the corresponding mutated nucleotides. These mutations at F87A and A264G were checked by DNA sequencing. After the incubation of the recombinant *E. coli*, *E. coli* cells were subjected to sonication. The supernatant containing P450BM3 was applied to an open column containing 50 mL of CelfineA-500 from TOYOBO (Osaka, Japan). After red-brown fractions containing P450BM3 were collected, the sample was applied to ion and gel filtration chromatographies. Purification level of P450BM3 was checked by SDS-PAGE. Furthermore, reduced CO difference spectra of WT and its mutants were measured to quantify P450 proteins.

The reaction solution contained 0-6.12 µM CB118 with a final concentration of DMSO at 0.1% (v/v), 1 µM purified WT, F87A or A264G, 100 µM PFCA (PFCA-C6, PFCA-C7, PFCA-C8, PFCA-C9, PFCA-C10, or PFCA-C11), 200 mM Tris-HCl (pH7.4), and 5 mM NADPH in a total volume of 0.5 mL. The reaction was started by adding NADPH, and the reaction solution was incubated for 3 h at 37°C with continuous shaking. Control experiments were demonstrated using the same reaction solution without NADPH or P450BM3. CB118 metabolites were extracted with four volumes of hexane twice and derivatized by methylation as described previously [6]. Quantification and identification of the metabolites were performed by high resolution gas chromatography and high resolution mass spectrometry (HRGC/HRMS) under the conditions described previously [2].

The docking models of WT with PFCA-C6, PFCA-C8, and PFCA-C10 were constructed by using the 3D structure of P450BM3 binding palmitoleic acid (PDB : 1FAG) as a template by the Surflex Dock of SYBYL-X2.1 (Tripos, St Louis, MO) as described previously [7]. Thereafter, docking models with CB118 were constructed by binding CB118 to those 3D models. An original cavity was defined as closed form of WT (PDB : 1FAG) removing palmitoleic acid. The docking models of F87A and A264G with PFCAs and CB118 were constructed as mentioned above.

Results and discussion

Four different hydroxylated pentachlorobiphenyls (OH-PeCBs, M2, M3, M4, and M5) by WT were produced dependent on the addition of NADPH. M3 was identified as hydroxylated PeCB at 4-position by the correspondence of its retention time with the standard. M2, M4, and M5 were not identified because retention times of all standards were not consistent with them. The production activity of M5 was the highest among metabolites. The reaction was accelerated by addition of PFCA-C6, PFCA-C7, PFCA-C8, or PFCA-C9 as decoy molecules (Figure 2). The production activity of M4 was the most accelerated among metabolites. When PFCA-C8 was used, production activities of alomost all of metabolites were the highest among PFCAs. In the previous study, the size of P450BM3's large substrate-binding cavity could be controlled by the addition of different length of PFCAs [4]. It has been reported that PFCA-C9 and PFCA-C10 were the most desirable decoy molecule in metabolisms of butane and cyclohexane, and propane by WT, respectively [4]. In addition, PFCA-C9 was the most appropriate decoy molecule in metabolism of benzene by WT [5]. These results show the appropriate combinations of decoy molecules and unnatural substrates to stay in the P450BM3 substrate binding cavity, suggesting that the total size of combination of a decoy molecule and an unnatural substrate was the same as the size of original substrates, long-chain fatty acids. The production activities of M5 were higher by adding PFCA-C6, PFCA-C7, PFCA-C8, and PFCA-C9 and lower by adding PFCA-C10 and

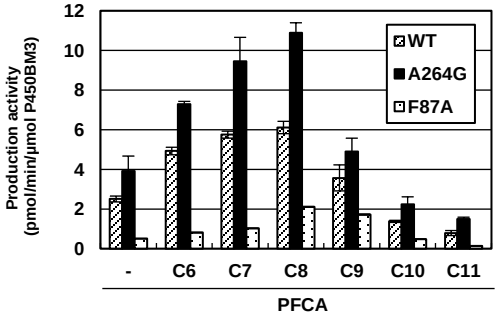

Figure 2 Production activities of hydroxylated pentachloro metabolites, M5 from CB118 by P450BM3 WT, A264G, and F87A with different length of PFCAs

PFCA-C11 than those not adding PFCAs (Figure 2). It is suggested that PFCAs with a long chain fully occupied the substrate-binding cavity, so CB118 cannot be accomodated in the reaction space above the heme.

Several factors to differentiate the production activities of four different metabolites by adding PFCAs were examined by the 3D docking models. According to the docking models of WT with PFCA-C6, PFCA-C8, or PFCA-C10, the substrate-binding cavity was smaller as the length of PFCAs was longer. Since PFCA-C6 with a short chain formed a larger substrate-binding cavity, it was too large to accommodate CB118 above the heme. In contrast, PFCA-C10 with a longer chain formed smaller substrate-binding cavity, so it was too small to settle CB118 in the cavity. Thus, the substrate-binding cavity with PFCA-C8 was the most suitable for positioning of CB118 to be metabolized.

In the substrate-binding cavity in WT with PFCA-C8, two possible conformations of CB118 were proposed; the conformations A and B show that the trichlorophenyl ring and dichlorophenyl ring are close to the heme, respectively (Figure 3). The crash values of conformations indicated that the conformation A was more stable than the B. The conformation of CB118 producing M5 was thought to be very stable because M5 was the most produced among metabolites. In the conformation A, 5-position of the trichlorophenyl ring was closest to the heme iron in any positions. The closest part to the heme iron was easily hydroxylated by P450s. Therefore, M5 was estimated as 5-OH-PeCB.

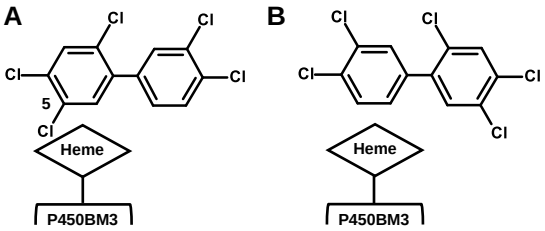

Figure 3 Possible conformation of CB118 in the substrate-binding cavity of WT with PFCA-C8
(A) Conformation A, (B) Conformation B

We constructed F87A and A264G because these amino acids positioned close to the heme enough to control the volume of the cavity. In particular, it was reported that F87A and A264G were much higher activities for oxidation of the polycyclic aromatic hydrocarbons, phenanthrene, fluoranthene, and pyrene, which were environmental pollutants as PCBs, than WT [8]. Furthermore, it was reported that F87A oxidized *p*-xylene and 4,4′-dimethylbiphenyl more effectively than WT [9].

Five different OH-PeCBs (M1, M2, M3, M4, and M5) by A264G were detected dependent on the addition of NADPH. Some of these metabolites (M2-M5) were the same ones produced by WT. Hydroxylation activities of CB118 by A264G were high using PFCA-C7 and PFCA-C8 and showed similar trends dependent on the length of PFCAs as shown in WT. M5 was more produced by A264G than WT (Figure 2). Comparing of the docking models of WT and A264G with PFCA-C8 and CB118, 5-position of trichlorophenyl ring approached closer to the heme in the A264G cavity than in the WT cavity by enlargement of a part of the cavity by substitution of an amino acid. Since close distance between the reaction position of CB118 and the heme iron brings a high activity, the production activities of M5 by A264G were higher than by WT.

Two different OH-PeCBs (M4 and M5) by F87A were detected dependent on the addition of NADPH. These metabolites corresponded to M4 and M5 by WT and A264G. The production activities of M5 by F87A were much lower than those by WT and A264G (Figure 2). It is thought that substitution of phenylalanine to alanine made the substrate-binding cavity much larger. Then, the F87A cavity was too large to accommodate CB118 stably. The decoy molecules PFCA-C8 and PFCA-C9 showed higher production activities than the other lengths of PFCAs. These results show that a longer PFCA was needed to anchor CB118 above the heme in the F87A cavity to compensate the disadvantageous space produced by substitution of an amino acid.

We expect that our results will be applied to the efficient bioremediation by constructions of more active P450BM3 mutants for PCB metabolism using the 3D docking models. At the same time, our results will be useful to reveal the metabolic fates of PCBs under the complex pollution with PFCAs by soil bacteria.

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